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Growth and Mitogenic Effects of Arylphorin In Vivo and In Vitro

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In insects, developmental responses are organ- and tissue-specific. In previous studies of insect midgut cells in primary tissue cultures, growth-promoting and differentiation factors were identified from the growth media, hemolymph, and fat body. Recently, it was determined that the mitogenic effect of a *Manduca sexta* fat body extract on midgut stem cells of *Heliothis virescens* was due to the presence of monomeric α-arylphorin. Here we report that in primary midgut cell cultures, this same arylphorin stimulates stem cell proliferation in the lepidopterans *M. sexta* and *Spodoptera littoralis,* and in the beetle *Leptinotarsa decemlineata*. Studies using *S. littoralis* cells confirm that the mitogenic effect is due to free α-arylphorin subunits. In addition, feeding artificial diets containing arylphorin increased the growth rates of several insect species. When tested against continuous cell lines, including some with midgut and fat body origins, arylphorin had no effect; however, a cell line derived from *Lymantria dispar* fat body grew more rapidly in medium containing a chymotryptic digest of arylphorin. Arch. Insect Biochem. Physiol. 64:63–73, 2007. Published 2007 Wiley-Liss, Inc.†

KEYWORDS: arylphorin, mitogen, growth, insect

INTRODUCTION

Ecdysteroids and juvenile hormones synchronize developmental changes within insect organs to bring about the broader developmental processes of the molt and metamorphosis. Thus, proliferation can occur in one tissue or cell type, while apoptosis is occurring in another (Truman and Schwartz, 1984). Ultimately, developmental responses are tissue- and organ-specific and the potential controls of cell development can also include local interactions such as cell-to-cell contacts, interactions with the extracellular matrix, and paracrine and autocrine factors. This is demonstrated in the development of the larval lepidopteran insect midgut. At each larval molt, a population of undifferentiated stem cells in the lepidopteran midgut differentiates to goblet and columnar phenotypes while intercalating between existing mature goblet and columnar cells present

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in the midgut epithelium (Baldwin and Hakim, 1991). Since the unique larval distribution pattern of these two cell types is maintained during this growth process, there must be local communication among the various cell types.

In vitro, lepidopteran midgut cells follow the same pattern of development as midgut cells in vivo, in that only stem cells multiply and subsequently differentiate to mature cell types; in vitro, however, mature cells die and are replaced by newly differentiated stem cells (Loeb and Hakim, 1996). To induce the stem cells to multiply in vitro, and thus increase the total number of cells, fat body tissue (Sadrud-Din et al., 1994) or an aqueous extract of fat body tissue (FBX) (Loeb and Hakim, 1996) was added to the culture medium. Fat body or FBX from *Heliothis virescens* (tobacco budworm), *Manduca sexta* (tobacco hornworm), or *Lymantria dispar* (gypsy moth) were equivalent in stimulating proliferation of midgut stem cells obtained from larvae of either *M. sexta*, *H. virescens* (Loeb and Hakim, 1996), or *L. dispar* (Dougherty et al., 2006).

Using cation-exchange chromatography, Blackburn et al. (2004) isolated a fraction of *M. sexta* fat body extract that contained a single peak of UV absorbance and stimulated the proliferation of midgut stem cells from *H. virescens* in vitro. This fraction, when analyzed by SDS-PAGE, produced two prominent bands of equal intensity with apparent molecular masses of 77 and 72 kDa. Subsequent fractionation of this active material by anion-exchange chromatography resulted in two peaks of UV absorbance, a small early eluting peak that stimulated stem cell proliferation and a large late eluting peak that was inactive. Analysis of these peaks by SDS-PAGE revealed that the smaller, active peak produced a single band of 77 kDa, while the larger peak produced bands of both 77 and 72 kDa. Edman degradation of four peptides from a tryptic digest of the smaller peak produced four sequences that were identical to internal sequences of *M. sexta* α-arylphorin (Willott et al., 1989). The active 77 kDa protein passed freely through a 100-kDa ultrafiltration membrane. Based on these results, the active factor was identified as monomeric α-arylphorin.

Arylphorin (originally termed manducin) is a hexameric protein composed of α- and β-subunits. It was first identified as a major constituent of larval and prepupal insect serum (Kramer et al., 1980) that functions as a source of the aromatic amino acids phenylalanine and tyrosine needed for cuticle tanning (Ryan et al., 1985). Synthesized primarily by fat body, arylphorin is secreted into the hemolymph, peaking at 30–40 mg/ml concentrations in the *M. sexta* prepupa (Kramer et al., 1980). Arylphorin is then resorbed by the perivisceral adipocytes and packaged in crystalline storage bodies (Haunerland, 1996). Arylphorin is produced throughout the larval instars, though apparently not during the molt, when mRNA for arylphorin is inhibited by high ecdysteroid titers (Webb and Riddiford, 1988). Yet there are no unequivocal larval functions for arylphorin (N. Haunerland, personal communication) despite the fact that the pattern of arylphorin production and secretion from fat body is suggestive of such a function.

Thus, the identification by Blackburn et al. (2004) of arylphorin as the active mitogen in fat body extract was unexpected. In order to confirm this activity and assess whether this activity is conserved across insect orders, families and genera arylphorin from a common source of fat body extract were assayed in primary/secondary insect cell culture and/or whole insects of different orders (Lepidoptera, Coleoptera, Homoptera), families within an order (Noctuidae, Sphingidae) and genera within the family Noctuidae (Heliothis, Spodoptera, Trichoplusia). This approach was expanded to test for an effect on established insect cell lines. Lastly, in order to determine whether the in vitro results had an in vivo counterpart, arylphorin was included in the diets of most of these same test insects. This feeding study addressed the additional question of whether the toxicity of orally administered fat body extract on the cotton leafworm Spodoptera littoralis, reported by Smagghe et al. (2001), can be attributed to arylphorin.

MATERIALS AND METHODS

Source and Preparation of Arylphorin

Fat body extract was prepared from the abdominal, "green," fat body of newly ecdysed *M. sexta*

pupae as described by Loeb and Hakim (1996). Subsequently, Blackburn et al. (2004) found that the mitogenic effect of *M. sexta* FBX on *H. virescens* midgut stem cells was due to small amounts of the α-arylphorin subunit that could be separated from the hexameric form of the protein. In this report, we refer to the crude mixture of hexameric arylphorin and monomeric α-arylphorin as "total arylphorin," the purified α subunit as "monomeric α-arylphorin," and a fraction lacking the free α-subunit "total arylphorin minus free α."

These were purified from FBX (Blackburn et al., 2004). Total arylphorin was isolated by diluting 5 ml of FBX to 50 ml with 20 mM sodium acetate (pH 4.75), and loading this mixture onto a 2.6 × 10 cm SP Sepharose FF column (Pharmacia; Uppsala, Sweden) equilibrated with sodium acetate buffer. After the UV absorbance of the effluent had stabilized, the column was eluted with a gradient of 0–500 mM KCl in the same buffer over a 25-min period at 4 ml/min. This procedure results in a single large peak of UV absorbance, composed of nearly pure arylphorin. The identity of the protein was confirmed by SDS-PAGE. Subsequently, small amounts of monomeric α- arylphorin were separated from this "total arylphorin" by anion exchange chromatography. Total arylphorin fractions were diluted 1:10 with 20 mM Tris-HCl (pH 8.6) and loaded onto a BioChrom Hydrocell NP10 DEAE column (7.8 × 75 mm). The bound material was then eluted with a gradient of 0–250 mM KCl in the same buffer at 0.5 ml per min. This step results in a small, early eluting, peak of monomeric α-arylphorin, and a much larger, later eluting peak of arylphorin composed of total arylphorin minus free α. Arylphorin was added to media, or diet in Tris-HCl buffer.

Primary Culture Growth Conditions and Proliferation Assays

Primary midgut cultures were prepared as described previously (Loeb and Hakim, 1996), with the exception that low concentrations of total arylphorin were substituted for FBX during experimental procedures. The effect of total arylphorin on *M. sexta* midgut stem cells was determined by assaying the percent of labeled nuclei in a 5-bromo-2′-deoxy-uridine (BrdU) incorporation assay (product no. 1299964; Roche Applied Science, Indianapolis, IN). This assay provides a measure of the proportion of cells that are actively synthesizing DNA, an early step in cell replication. Cells, including all midgut cell types, were collected from cultures grown in complete media containing FBX, then resuspended at 1×10^5 cells/ml in fresh media lacking FBX. The cells were allowed to incubate for 4 h to condition the medium (CM). One microliter BrdU from the kit was added to each milliliter of culture medium followed by selected amounts of total arylphorin, FBX, or water (control). Thirty-six hours later, BrdU incorporation into cell nuclei was determined using an antibody-based assay.

For *S. littoralis* and the Colorado potato beetle, *Leptinotarsa decemlineata*, cell proliferation was measured by counting the cells in a culture well 3 days following application of arylphorin. *S. littoralis* midgut stem cells were exposed to the total arylphorin, monomeric α-arylphorin, and the "total arylphorin minus free α" fractions, while *L. decemlineata* cultures were exposed to the monomeric α-arylphorin.

Continuous Culture Growth Conditions and Assays for Proliferation

Cell lines used in the in vitro studies included IPLB-HvT1 (*H. virescens*), IPLB-LdFB, IPLB-LdElta, IPLB-LdEp (*L. dispar*) (Lynn et al., 1988), IAL-TND1 (Lynn et al., 1982), TnCL1 (*T. ni*; cabbage looper) (a clone of TN-368; McIntosh and Rectoris, 1974), IPLB-Sf21 (*Spodoptera frugiperda*; fall armyworm) (Vaughn et al., 1977), BCIRL-HzAM1 (McIntosh et al., 1981) BCIRL-HzFB33 (Kariuki et al., 2000), RP-HzGUT-AW1 (*Helicoverpa zea*; corn earworm) (Goodman et al., 2004), CF-203 (*Choristoneura fumiferana*; spruce budworm) (Sohi et al., 1993), and BCIRL-Lepd-SL1 (*L. decemlineata*) (Long et al., 2002). All cell lines were grown in Ex-Cell 400 or 401 medium (JRH Biologicals, Lenexa, KS). The medium was supplemented with either 5% fetal bovine serum (FBS) for HvT1 and TND1 cells or 10% FBS for HzAM1, HzFB33, HzGUT-AW1, Lepd-SL1, and TnCL1.

Preliminary screening was performed on each cell line by inoculating 24-well tissue culture plates (Falcon®) with cells in 0.5 ml medium at four cell densities equivalent to 25, 50, 100, and 200% normal seeding density or in 0.2 ml at 5×10^2 or 5×10^3 cells/well. After 4 or 24 h, total arylphorin or chymotrypsinized total arylphorin (prepared by incubating arylphorin 12 h with bead-bound chymotrypsin [Sigma] and removing the chymotrypsin-beads by centrifugation) was added to cells at 0.025, 0.05, 0.1, 0.2, 0.3 or 0.4 μg/ml. Cells were observed at 24 h for morphological effects and at 3 or 5 days post-treatment for cell proliferation.

Based on these initial tests, the LdFB cells were subjected to further study. Cultures were initiated in 25 cm^2 Falcon® tissue culture flasks with 2.8×10^4 cells/ml in 4.0 ml of Ex-Cell 400 medium. Triplicate individual cultures were treated with total arylphorin or chymotrypsinized total arylphorin at a final concentration of 0.025, 0.05, or 0.1 μg/ml and three cultures were left as untreated controls. Cultures were incubated at 26°C and counted in situ at 48-h intervals. Cell numbers for each culture were estimated by counting the number of viable cells in five predetermined microscope fields.

Insect Rearing Conditions, Diets, and Assays for Growth

Eggs of *M. sexta* were obtained from a colony maintained at the University of Arizona. Larvae were raised on artificial diet (Bell and Joachim, 1976) at 25°C, under 16:8 L:D. Larvae of *S. littoralis* were grown at 23°C, 16:8 LD, on artificial diet as described in Smagghe et al. (2000). *T. ni* eggs were obtained from Bio-Serv (Frenchtown, NJ), hatched in paper cartons, and grown on a Bio-Serv wheat and soybean based diet, item no. F9772. One to three neonates were grown per well on diet, in 24-well culture plates The larvae were reared in the dark at 28–29°C, 45–50% humidity. Larvae of *L. decemlineata* were reared on an artificial diet in sealed cups (Gelman et al., 2001).

Sweet potato whiteflies, *Bemisia tabaci* (Biotype B), were grown on a variety of plants in climate-controlled insect growth chambers (26 ± 2°C, 16:8 L:D, 60% RH) (Gelman et al., 2002). One day prior to hatch, eggs were collected, surface sterilized and placed on membranes in sterile rearing chambers (Jancovich et al., 1997). Filter sterilized (0.22 μM) aqueous diet contained 15% sucrose, 5% yeast extract, and total arylphorin (1, 10, and 50 μg/ml final concentration), or an equivalent amount of water, in controls. After 20 to 30 days, 4th instar and adult whiteflies were counted and percent survival calculated. Because the weight of individuals was too small for easy determination, length and width were assessed as measures of growth, using an optical micrometer.

Statistics

Analysis of variance (ANOVA) followed by Tukey's comparison of means test ($\alpha = 0.05$) was used to determine significant differences among means.

RESULTS

Effect of Arylphorin on Primary Midgut Cultures

M. sexta primary cultures containing approximately 3,000 to 4,000 cells were grown and stained for BrdU incorporation. Thirty-six hours later, groups of 200 cells were counted to assess BrdU incorporation. More than twice as many cells were labeled with BrdU in cultures that contained total arylphorin (100 ng/ml) than in those that contained 20 μl/ml FBX in the medium (our standard culture condition) or in cultures grown without either total arylphorin or FBX, respectively. Incorporation peaked at a total arylphorin concentration of 100 ng/ml. At lower and higher concentrations, BrdU incorporation dropped off sharply (Fig. 1).

In cell-counting experiments, total arylphorin, purified monomeric α-arylphorin, and total arylphorin minus free α fractions were tested for mitogenic activity in midgut cultures from *S. littoralis*. While the monomeric α-arylphorin appeared to have only slightly higher activity than the total arylphorin fraction, the total arylphorin minus free α fraction was inactive at all doses tested (Fig. 2). The responses of *L. decemlineata* midgut cells to

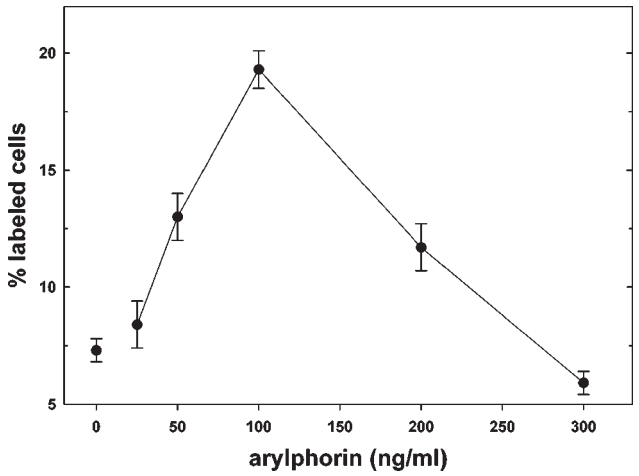

Fig. 1. The effect of total arylphorin on BrdU incorporation in *M. sexta* primary culture prepared from fourth instar midgut. Cells were grown in medium containing 1 µl/ml BrdU along with 5 different concentrations of arylphorin or conditioned medium ("0") alone. At the end of 36 h, the cells were assessed for labeling. Each point represents the mean percent labeled cells ± S.E. of four or six replicate 200 cell samples.

monomeric α-arylphorin alone are shown in Figure 3. In all three species, the number of cells in culture increased in response to increasing concentrations of either total arylphorin or monomeric α-arylphorin. The stimulatory effect reached a maximum within a specific range of concentrations, then this effect declined with further increases in concentration.

Effect of Arylphorin on the Proliferation of Cells in Continuous Cell Lines

No effects were observed with cell lines treated with total arylphorin. However, the fat body cell line, IPLB-LdFB, did respond with slightly increased growth rates when treated with a chymotryptic digest of total arylphorin. This was done because the mature cells of midgut express endogenous proteases, which the lines tested probably do not. Cell proliferation was maximal and significantly different from controls on days 5 and 9 at a concentration of 50 ng/ml digest; however, at higher and lower doses of the chymotryptic digest, no stimulatory effect was observed (Fig. 4). Other lines

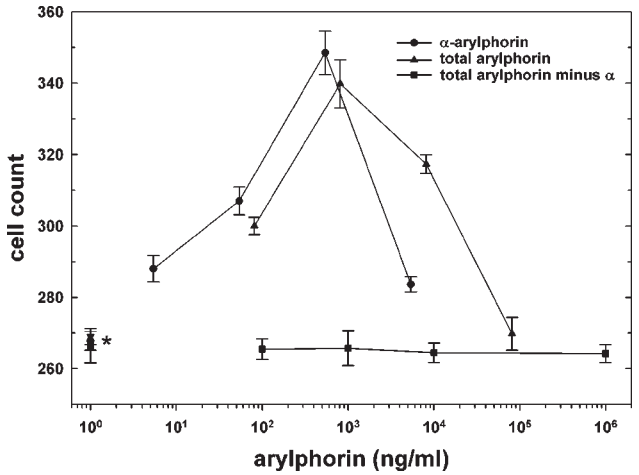

Fig. 2. The effect of arylphorin on stem cell number in *S. littoralis* primary culture prepared from pharate sixth instar midgut. Cells were grown for 3 days in conditioned medium (control), or in medium containing concentrations of monomeric α-arylphorin, total arylphorin, or total minus α-arylphorin. Each point represents the mean ± S.E. of 6 replicates. The leftmost points, marked with an asterisk, are the controls for each of the preparations.

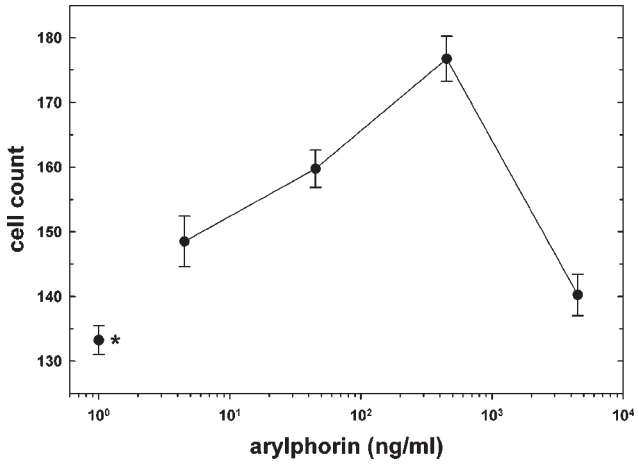

Fig. 3. The effect of arylphorin on stem cell number in *L. decemlineata* primary culture prepared from pharate fourth instar midgut. Cells were grown for 3 days in conditioned medium alone (control), or in conditioned medium containing concentrations of monomeric α-arylphorin. Each point represents the mean ± S.E. of 4 replicates. The leftmost point, marked with an asterisk, indicates the control response.

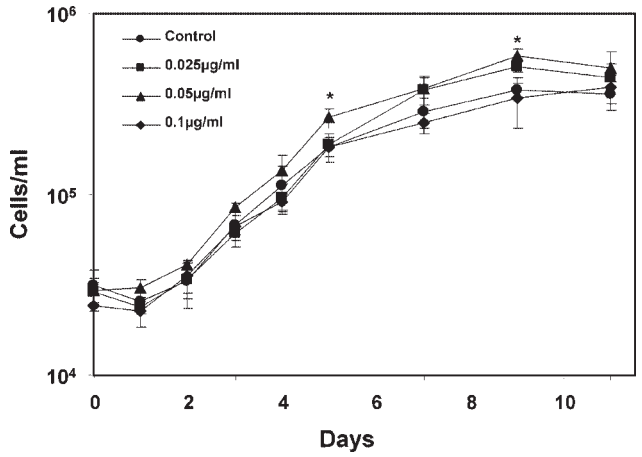

Fig. 4. Growth curves for IPLB-LdFB cells grown in the presence of chymotrypsinized aryphorin concentrations and untreated controls. Points are the means ± S.D. of three replicates. As determined by Tukey's comparison of means ($\alpha = 0.05$), cultures treated with 0.05 µg/ml had significantly greater cell numbers on days 5 and 9 as compared to control cultures.

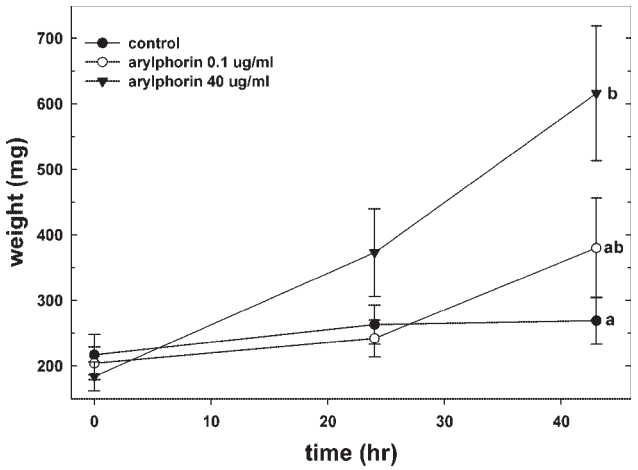

Fig. 5. The effect of total arylphorin on weight gain of 4th instar *M. sexta*. Newly molted 4th instar tobacco hornworms were reared for 44 h on control diet and diets containing two different concentrations of arylphorin. Each value represents the mean ± S.E. of the weights of at least 6 larvae. A one-way ANOVA followed by Tukey's comparison of means test was used to assess significant differences among treatments of interest. Points labeled with different letters are significantly different ($\alpha = 0.05$).

tested with this same digest showed no effect (IPLB-HvT1, IPLB-LdElta IPLB-Sf21AE, IAL-TND1).

Effects of Total Arylphorin When Administered Through Feeding

All species except the *B. tabaci* showed evidence of increased weight or size in response to the addition of total arylphorin to their diets (Figs. 5–8 and Table 1). When arylphorin was administered at 10 µg/ml of diet to *B. tabaci*, there was a significant decrease in both length and width as compared to controls (Table 1). In contrast, Figures 5 and 6 demonstrate that diets containing total arylphorin caused a progressive increase in larval weight for *M. sexta* and *S. littoralis* so that with increasing time, the differences in mean weight between control and experimental larvae were more pronounced. Values for the final weights attained were significantly higher than control values for both 0.1 or 40 µg/ml diet-fed *S. littoralis* (Fig. 6) and for 40 µg arylphorin/ml diet-fed *M. sexta* (Fig. 5). In Figures 7 (*L. decemlineata*) and 8 (*T. ni*), the mean insect weights are shown at the end of the experiments. For both insects, the maximum

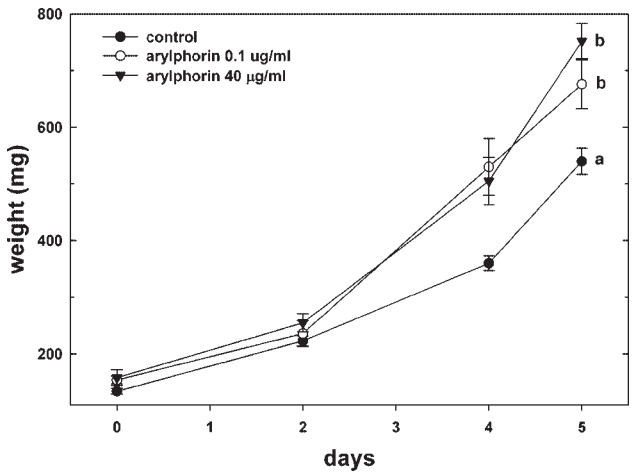

Fig. 6. The effect of total arylphorin on weight gain of 6th instar *S. littoralis*. Sixth instar cotton leafworms were reared on control diet and diets containing two concentrations of total arylphorin for 5 days. Each value represents the mean weights ± S.E. of at least 6 larvae. A one-way ANOVA followed by Tukey's comparison of means test was used to assess significant differences among treatments of interest. Points labeled with different letters are significantly different ($\alpha = 0.05$).

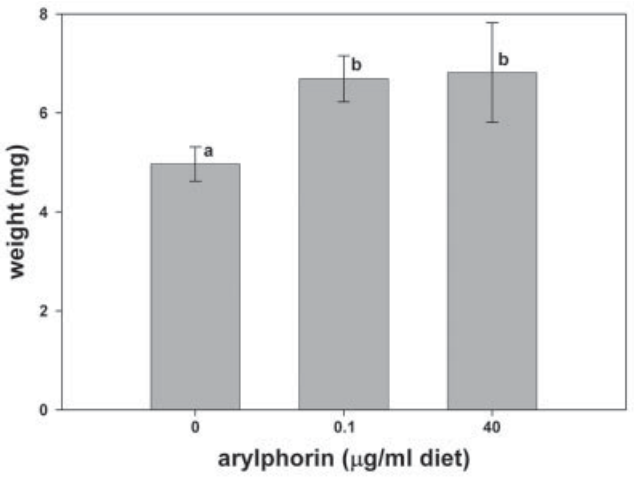

Fig. 7. Weights of 2nd instar *L. decemlineata* reared on a control diet and on diets containing two concentrations of total arylphorin. Weight was determined after 44 h of feeding. Each bar represents the mean weight (± S.E.) of at least 6 larvae. A one-way ANOVA followed by Tukey's comparison of means test was used to assess significant differences among the treatments. Points labeled with different letters are significantly different ($\alpha = 0.05$).

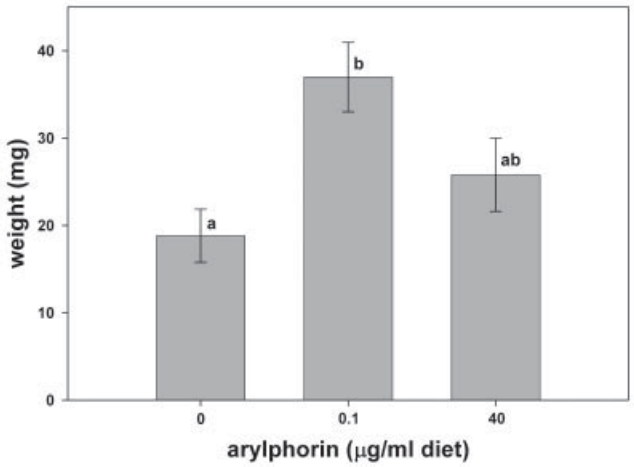

Fig. 8. Weights of *T. ni* neonates reared on a control diet and on diets containing two concentrations of total arylphorin. The diets of control animals contained a volume of water equal to the volume of arylphorin added to experimental diets. Weights were determined after 6 days of feeding, when most larvae were in the 6th instar. Each bar represents the mean weight (± S.E.) of at least 16 larvae. A one-way ANOVA followed by Tukey's comparison of means test was used to assess significant differences among the treatments. Points labeled with different letters are significantly different ($\alpha = 0.05$).

TABLE 1. The Effect of Arylphorin on the Body Length and Width Attained by 4th Instar Nymphs of *B. tabaci**

Treatment	Body length (mm) ± SE	Body width ± SE
Control	0.67 ± 0.0057^{ab}	0.4439 ± 0.0045^{a}
1 µg/ml	0.68 ± 0.0061^{a}	0.4521 ± 0.0053^{ab}
10 µg/ml	0.64 ± 0.0052^{c}	0.4283 ± 0.0049^{c}
50 µg/ml	0.66 ± 0.0059^{bc}	0.4351 ± 0.0047^{bc}

*Each value represents the mean ± SE of a least 45 separate determinations. Means with the same superscript letters were not significantly different ($\alpha = 0.05$).

weights attained are noticeably higher for experimental as compared to control larvae. For *L. decemlineata* (Fig. 7), increases in weight after treatment with 0.1 or 40 µg/ml arylphorin, compared with controls, were significant at $P = 0.1$. For *T. ni* (Fig. 8), treatment at 0.1 µg/ml, but not 40 µg/ml, caused a significant increase in body weight as well.

DISCUSSION

Insect fat body has been shown to stimulate *Galleria mellonella* imaginal disc growth in culture (Richman and Oberlander, 1971), to stimulate growth and development of *H. virescens* genital imaginal tract (Loeb and Hakim, 1991), and to stimulate proliferation in primary cultures of *M. sexta* midgut epithelium (Sadrud-Din et al., 1994). Fat body extract can replace fat body as the mitogen in primary cultures of *M. sexta* midgut epithelium (Loeb and Hakim 1996). Aqueous extracts of fat body have also been shown to support the growth and development of imaginal wing discs of several lepidopteran species (Benson et al., 1974; Smagghe et al., 2001) cultured in vitro.

Fractionation of conditioned medium from Drosophila imaginal disc cultures has identified a family of five polypeptide growth factors that originate from fat body, among other sources, and stimulate imaginal disc growth in cooperation with insulin (Kawamura et al. 1999). They have similarity to chitinase (52 kDa) and like arylphorin are active in the nanomolar range. A sixth member of this family from the cabbage moth *Mamestra brassicae* was recently reported to stimulate proliferation in a fat body and a hemocyte-derived lepidopteran cell line under insulin-free conditions

(Zhang et al., 2006). Another distinct factor, of neural origin, bombyxin (4,500–5,000 Da), is an insulin-like peptide that functions as a growth factor for wing imaginal discs in the buckeye butterfly, *Precis coenia* (Nijhout and Grunert, 2002) and for mixed and stem cell primary/secondary cultures of midgut cells derived from *M. brassicae* and the tobacco budworm *H. virescens* (Goto et al, 2005). Bombyxin works synergistically with FBX and thus would appear to complement the arylphorin activity identified from FBX.

Monomeric α-arylphorin stimulated the proliferation of midgut stem cells of *H. virescens* in vitro (Blackburn et al., 2004); this molecule consisted of free α- subunits that were chromatographically separated from the hexameric form of the protein; α-arylphorin accounted for 5–6% of the total arylphorin (based on peak area). In this report, results from *S. littoralis* midgut cultures confirmed that the active component was the monomeric α-arylphorin while the total arylphorin minus free α was found to be inactive at all concentrations tested.

The current study demonstrates the breadth of the arylphorin effect. This activity on such diverse insects further suggests that the midgut cell response to arylphorin has been conserved over evolution. Total arylphorin increases the numbers of midgut stem cells in the moth species *M. sexta, S. littoralis, T. ni*, and the beetle *L. decemlineata*. There even appears to be some potentiation of the effect of α-arylphorin by the intact hexamer. Blackburn et al. (2004) reported that α-arylphorin maximally stimulated *H. virescens* stem cell proliferation at a concentration of 125 ng/ml. Subsequently, it was found that the total arylphorin fraction exhibited maximal activity on *H. virescens* cultures at similar concentrations (data not shown), despite the fact that the monomeric α-arylphorin present was less than 8 ng/ml (5–6% of the total arylphorin). A similar pattern is seen in the midgut cell culture results for *S. littoralis*; thus the activity of the total arylphorin fraction was greater than could be accounted for by the monomeric α-arylphorin it contained alone.

Studies using FBX in midgut cultures have demonstrated that FBX has activity as a mitogen. While arylphorin appears to be the molecule in FBX responsible for this activity in our cultures, it is possible that FBX contains additional growth factors, such as an IDGF, not yet identified. A potential role for arylphorin in suppressing cell death, though unlikely, remains unknown The mechanism by which cell proliferation is increased has not been determined. Possibilities include a shortening of the cell cycle, recruitment of a usually nondividing stem cell population into the cycle or a shift in stem cell proliferation from one where stem cells divide and give rise to one daughter that differentiates and another that retains division capacity to a geometric proliferation system where stems divide to primarily more stems.

Smagghe et al. (2003) reported that *S. littoralis* and *L. dispar* larvae fed FBX exhibited decreased growth rates, premature molting, and hyperplasia of the midgut. From the data presented in this study, it is clear that arylphorin alone is not sufficient to reproduce those results previously reported for FBX. When total arylphorin was added to artificial diets fed to *M. sexta, S. littoralis, T. ni*, and *L. decemlineata* larvae, higher rates of growth were observed. Therefore, the FBX may contain inhibitory substances not present in total arylphorin. In contrast, *B. tabaci* nymphs fed total arylphorin exhibited a decrease in length and width at a dose of 10 μg/ml as compared to controls. Proteases are present in *B. tabaci* gut, i.e., ingested leaf proteins are digested into their component amino acids (Salvucci et al., 1998). It may be that the sucking mode of feeding, the unique gut structure that includes a filtration chamber (which facilitates the diffusion of liquids from the foregut to the hindgut; Weber, 1935), and/or the nature of the gut environment is responsible for the differences in total arylphorin effects observed in the whitefly as compared to the other insects examined. This lack of effect is not, however, due to pH differences since the lepidopteran larvae tested had alkaline midgut pH's while the coleopteran larvae (*L. decemlineata*) midgut pH is neutral.

The mechanism by which arylphorin stimulates increased cell proliferation and weight gain is not known. Since arylphorin was originally described as a storage protein that provides a source of aro-

matic amino acids for the pharate adult, the effect could simply be nutritional, with the compound providing micronutrients that are in limited supply. This is unlikely, however, since our results from in vitro studies demonstrate a maximum effective concentration of arylphorin (100 ng/ml in primary midgut cultures from *M. sexta*), with significantly less stimulation at higher concentrations. This result is typical and has been shown previously for arylphorin concentrations on *Heliothis virescens* midgut culture by Blackburn et al. (2004) and for the effect of bombyxin on *Mamestra brassicae* midgut culture (Goto et al., 2005). Furthermore, the basic tissue culture medium for our primary cultures is itself a rich complete growth medium that includes Grace's medium, vitamin premix, 20-hydroxyecdysone, and fetal calf serum; Grace's culture medium itself contains 50 μg/ml tyrosine and 150 μg/ml phenylalanine (Cambrex Inc. formulation, technical specification sheet). Thus, the contribution of the added arylphorin to levels of these amino acids is negligible.

The continuous cell lines raised in serum-free media did not benefit from the addition of arylphorin; these included the RP-HzGUT-AW1 and CF-203 lines originally established from *H. zea* and *C. fumiferana* midgut, respectively. However, the culture medium in this and several of the other lines that we have tested were already supplemented with FBS, which is a known source of vertebrate growth factors. Even serum-free media may contain proteins, and the growing cells themselves contribute to the medium. While only circumstantial evidence exists that the vertebrate factors in FBS are active on insect cells, any cells capable of growth as established lines are likely to be using these factors or providing their own growth factors. That several continuous cell lines produce growth factors constitutively was demonstrated by co-culture with genital tract in vitro (Loeb and Lynn, 1993). The fat body–derived LdFB cell line that benefited from the chymotryptic digest of arylphorin in our study typically grows slightly slower in serum-free medium, so the arylphorin may be supplementing their autocrine factors. The fact that this growth stimulation was only observed with the chymotryptic digest of arylphorin is consistent with the results obtained by feeding arylphorin to insects, since tryptic enzymes are in the insect gut and thus should affect orally presented arylphorin.

Arylphorin added to the diet at microgram per milliliter diet concentrations lead to an increase in weight or size in all but *B. tabaci*. While the instars measured and times when measurements were carried out differed (they reflected standard practices in the individual cooperating laboratories), it is remarkable that, with the exception of *B. tabaci*, they all showed the same result: an increase in weight due to addition of arylphorin to the diet. The basis of this increase is not yet known.

To determine whether arylphorin plays a role as a mitogen in midgut development in vivo will require more research. Arylphorin is certainly available to the midgut; it is present in the larval hemolymph during intermolt periods (Kramer et al., 1980; Telfer et al., 1983; Karpells et al., 1990), and the midgut of the hesperiid *Calpodes ethlius* apparently produces arylphorin and secretes the protein basally, into the hemolymph (Palli and Locke, 1987). Although arylphorin was not expressed in *M. sexta* midgut (Webb and Riddiford, 1988), these experiments were performed only on day 2 of the 5th instar (corresponding to the peak of arylphorin expression by fat body). It is noteworthy that the concentration of arylphorin that is optimum for the stimulation of stem cell proliferation is a small fraction of the concentration present in the hemolymph of lepidopteran larvae (Kramer et al., 1980, Telfer et al., 1983; Karpells et al., 1990). Based on results reported here, intermolt hemolymph concentrations of arylphorin are so high they would inhibit the multiplication of midgut stem cells. Perhaps this is why stem cell proliferation occurs just prior to the molt, when arylphorin levels are dramatically reduced from peak levels. Additionally, access of arylphorin from the hemolymph may be limited. Perhaps arylphorin receptors governing stem cell mitogenesis are on the apical cell surface of the midgut epithelium, which is separated from the basal domain, and the hemolymph, by extensive septate junctions (Baldwin and Hakim, 1987; Baldwin et al., 1993).

These midgut junctions substantially block the paracellular flow of large molecules to the apical surface; thus, structural separation may play a role in reducing the availability of arylphorin to the apical midgut surface (Baldwin and Hakim, 1987; Baldwin et al., 1993). This hypothesis is also consistent with the results from feeding experiments, where relatively small amounts of arylphorin added to the diet were effective in stimulating weight gain, while higher concentrations already present in blood, didn't enhance weight gain above the levels observed for the orally presented lower concentrations. The orally presented arylphorin would reach the apical surface of the midgut, bypassing any structural or chemical barriers blocking access to the apical surface by arylphorin in the blood. Once in the gut lumen, the arylphorin is certainly exposed to the luminal environment including proteases such as chymotrypsin. This luminal environment is expected to be similar to that in primary/secondary cultures since midgut cultures contain mature digestive midgut cells and goblet cells.

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